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Comparison of Line Probe Assay (LIPA) and Sequence Analysis for Detection of HIV-1 Drug Resistance

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The identification of HIV strains that are resistant to antiretroviral drugs, which emerge during a patient's therapy or are already present in infected individuals prior to treatment, is of increasing importance for the clinical management of HIV-infected persons. Two different methods were compared for the genotypic detection of resistance development in the HIV-1 reverse transcriptase (RT) gene, the commonly used sequence analysis, and the commercially available RT-line immunoprobe assay (LIPA), which can detect mutations at six separate codons of the RT gene, which are known to confer resistance to certain nucleoside inhibitors. Eighty serum samples from HIV-1–infected persons, some of whom were receiving antiretroviral therapy, were investigated in parallel by sequencing as well as by LIPA. LIPA results agreed with sequence data in the vast majority of the cases. However, in 40% of the samples, LIPA failed to yield evaluable results for one or more of the codon positions. In particular, LIPA detection rate was low at codon 41 (75%), whereas at codons 69/70, 74, 184, and 215 results were obtained from 90%–95% of the samples. A number of mutations in the vicinity of the respective codons were detected by sequencing, and these may have been responsible for the LIPA hybridization failure. There remained a number of samples, however, where no explanation for the lack of hybridization could be derived from sequence data. Our results indicate that the use of the LIPA does not eliminate the need for sequence analysis for detection of drug-resistant HIV strains. ***J. Med. Virol. 57:283–289, 1999.***

KEY WORDS: HIV; drug resistance; LIPA; sequence analysis

INTRODUCTION

Treatment of HIV infection by antiretroviral drugs inhibiting the viral reverse transcriptase (RT) or the HIV protease leads, especially when applied as combination therapy, to a significant increase in survival time and to the improvement of the quality of life of infected persons [Hammer et al., 1996; Carpenter et al., 1997]. The success of the antiviral treatment is, however, often impeded by the emergence of drug-resistant HIV strains in the course of therapy [Larder et al., 1989]. A number of specific mutations have been described so far, which arise in the reverse transcriptase and in the protease gene during therapy and which cause a significant decrease in antiviral drug efficiency [Larder and Kemp, 1989; St. Clair et al., 1991; Fitzgibbon et al., 1992; Tisdale et al., 1993; Schinazi et al., 1996].

The identification of drug-resistant HIV variants developing in the patients during the course of therapy is becoming increasingly important. When failure of the therapy becomes evident by an increase of the patient's viral load [Saag et al., 1996; O'Brien et al., 1997], screening for drug resistance can be helpful and may lead to a rapid and specific change of the therapeutic scheme according to the viral mutation.

Virus strains with mutations in the RT gene that confer drug resistance have also been repeatedly observed in patients who had never received any antiretroviral therapy [Erice et al., 1993; Rubio et al., 1997]. This is probably due to infection with drug-resistant HIV strains. Therefore, it will be increasingly important to know a patient's resistance profile, even before the first anti-HIV treatment is initiated.

Information about viral resistance to individual antiretroviral drugs could be gained so far only by time-

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consuming phenotypic analysis [Japour et al., 1993] or by sequence analysis. Alternative methods applied for HIV genotyping of the RT gene have also been described [Larder et al., 1991; Kaye et al., 1992; Eastman et al., 1995; Frenkel et al., 1995]. These, however, suffer mostly from the fact that one test has to be performed for each single mutation, and therefore this approach seems not practicable for the analysis of a variety of different mutations. Recently, a line immunoprobe assay (LIPA) was developed and is now commercially available. This allows the detection of certain mutations in the RT gene of HIV-1 that confer resistance against different nucleoside inhibitors (NIs) [Stuyver et al., 1997]. In this assay-biotinylated PCR fragments of the viral RT gene are generated and hybridized to immobilized oligonucleotide probe panels, which cover six of the critical RT gene regions, where mutations influence the sensitivity to NIs. With this test system, mutations of codon 41,70 and 215, responsible for development of zidovudine (AZT) resistance, as well as mutations in codon 69, 74, and 184, associated with resistance to ddC, ddI, and 3TC, respectively, can be detected in one test run, which is a clear advantage over other alternative genotyping approaches.

In the present study we compared the efficiency of this LIPA to that of sequence analysis for monitoring HIV-1–infected patients, who came to AIDS clinics for routine check-ups. The data showed that sequence analysis is still superior to this alternative genotyping method, mainly because the LIPA yielded the complete pattern of hybridization results necessary for evaluation of the resistance genotype in only about 60% of the cases.

MATERIALS AND METHODS

Patient

Seventy-six serum samples from 74 adult HIV-1–positive patients and 4 sera from 4 vertically infected children were investigated in the study. The HIV-1 infection had been determined in all patients by detection of HIV-specific antibodies by Western blot (Sanofi Pasteur, France). Eighteen of the patients had never been treated with antiretroviral therapy; the others were undergoing different therapeutic schemes.

LIPA

The RT amplification as well as the LIPA were performed exactly according to the test protocol provided and recommended by Innogenetics. After the HIV RNA isolation, an RT-nested PCR was performed with the biotinylated primers included in the test kit. The amplified fragments were then denatured and incubated with the LIPA membrane strips, on which the oligonucleotide probes were fixed. After hybridization, the colorimetric reaction was performed. The colored lines, which became visible on the strips, were interpreted according to the instructions of Innogenetics.

The LIPA results were judged as wild-type (WT) or as mutant (MU) when a clearly visible line was present either at the place where the WT probes or where the MU probes of a codon were fixed, as indicated in the LIPA protocol. When a WT and an MU line was visible simultaneously at the same codon, it was assumed that for this codon a mixture of WT and MU strains was present in the sample. When no reactivity was observed with the WT and the MU probes at a particular codon, the result was considered indeterminate for this codon, while the results of the other codons were read independently.

Sequence Analysis

For sample preparation and amplification, 500 μl of the serum samples was centrifuged for 1 hr at 17,000 g at 4°C. The pellet was resuspended in 50-μl ddH_2O; 150 μl of TRIzol was added and the sample was incubated for 5 min at room temperature. Then the solution was centrifuged for 15 min at 12,000 g and the supernatant was mixed with 20 μl of a solution containing dextran T5OO at a concentration of 1 μg/μl and then 100 μl of isopropanol was added. After centrifugation, the pellet was washed with ice-cold EtOH, dried, and resuspended in 10 μl of ddH_20. cDNA synthesis was performed at 42°C in a solution containing 10 μl of the purified sample, 5 μM of random hexamers (Perkin-Elmer, Norwalk, CT), 200 U of M-MLV Reverse Transcriptase (GIBCO-BRL, Bethesda, MD), 1 × RT buffer (GIBCO-BRL), 0.25-mM nucleotide mix (Pharmacia, Piscataway, NJ), and 40-U RNAse inhibitor (Boehringer Mannheim, Mannheim, Germany). Then a PCR was carried out with 10 μl of the RT reaction in a solution containing 1 × PCR buffer (Perkin Elmer), 2-mM $MgCl_2$, 0.2 mM of each dNTP (Pharmacia), 2 U of Taq polymerase (Perkin Elmer), and 2.5 pmol of the primers RT-9 5′-GTACAGTATTAGTAGGACCTA-CACCTGTC-3′ and RT-12 5′-ATCAGGATG-GAGTTCATAACCCATCCA-3′ [Stuyver et al. 1997]. The cycling conditions were 1 min at 94°C, followed by 40 cycles of 30 sec at 94°C, 30 sec at 57°C, 30 sec at 72°C, and a final 5 min at 72°C incubation step.

Two μl of the PCR solution were subjected to a nested PCR assay under identical conditions, using the nested primers RT-1 5′-CCAAAAGTTAAACAATGGC-CATTGACAGA-3′ and RT-4 5′-AGTTCATAAC-CCATCCAAAG-3′ [Stuyver et al. 1997], where 35 cycles were performed. The PCR products were analyzed on a 3% agarose gel.

The amplified fragments were purified using Chroma Spin Columns-100 (Clontech, Palo Alto, CA) and then subjected to ethanol precipitation. The sequencing reaction was carried out with the primers RT-1 and RT-4, using the Dye Terminator Cycle Sequencing Kit from PE Applied Biosystems (Foster City, CA). The sequence analysis was performed on a 373 DNA sequencer (Applied Biosystems). Sequence comparisons were performed with the aid of the Perkin Elmer Abi Prism AutoAssembler program.

TABLE I. Comparison of the Results Obtained From Genotypic HIV Drug Resistance Testing of 80 HIV-1–Infected Patients by Sequence Analysis and LIPA

	LIPA results			
Sequence data	WT	MU	W/M[a]	No result
Codon 41				
WT (n = 51)	43	0	0	8
MU (n = 25)	0	14	1	10
W/M[a] (n = 4)	1	0	1	2
Codon 69/70				
WT (n = 67)	54	1	9	3
MU70 (n = 12)[b]	0	11	0	1
MU69 (n = 2)[b]	0	2	0	0
Codon 74				
WT (n = 78)	71	0	0	7
MU (n = 2)	0	1	0	1
Codon 184				
WT (n = 44)	39	0	1	4
MU (n = 35)	0	33	0	2
W/M (n = 1)	0	0	1	0
Codon 215				
WT (n = 54)	51	0	0	3
MU (n = 23)	0	19	0	4
W/M (n = 3)	1	0	2	0

[a]W/M denotes a combination of wild-type (WT) and mutant (MU) virus, when either strain was detectable at a similar intensity or when a smaller fraction of mutant strains was present in a larger wild-type population. Samples with higher signals from mutant than from wild-type virus were included in the mutant group.
[b]In one case, a double mutation at codons 69 and 70 was detected.

RESULTS

Comparison of LIPA Results to Sequencing Data

First, the LIPA and sequence analysis of the RT gene were compared, when applied for detection of drug resistance mutations in patients' serum samples. Eighty serum samples from persons infected with HIV-1 were investigated in parallel by both assays and results are presented in Table I. These data indicate that in those cases where the LIPA yielded evaluable results, they agreed with the sequence data. There was, however, one exception at codon 70, where the LIPA exhibited an R70 mutation, while the gene analysis indicated a wild-type K70 sequence in this region. Follow-up samples from the same patient taken 2 and 3 months later indicated a wild-type sequence in codon 70 by sequence analysis as well as by the LIPA.

In 15 serum samples, the LIPA indicated the simultaneous presence of wild-type and mutant virus (Table I). In nine cases a faint hybridization signal for the R70 mutant was observed in addition to a strong signal with the K70 wild-type sequence. In all of these cases, however, sequence analysis showed the wild-type sequence only. Two of these samples were derived from patients in whom an antiretroviral therapy had never been applied. Thus, it cannot be excluded that in at least those two cases the R70 band in the LIPA is nonspecific. As shown in Table I, there were eight other cases where the LIPA or the sequencing indicated a combination of wild-type and mutant sequences, i.e., at codon 41, 184, or 215. In four of these cases, the mixture of wild-type and mutant strains was detected by both methods.

The data summarized in Table I further indicate that a relatively large number of indeterminate results were obtained by LIPA. The lowest reactivity with the LIPA probes was observed at the codon 41 region of the RT gene. Only 75% of the serum samples gave a positive hybridization signal with either the wild-type or one of the two mutant probe sequences. In contrast, the hybridization to the immobilized oligonucleotides covering the wild-type or mutant codons 69/70, 74, 184, and 215 of the HIV-1 RT gene yielded positive results in 95%, 90%, 93%, and 91% of the samples, respectively.

It was observed that positive hybridization signals at codon 41 were frequently more faint than those obtained by hybridization with the other codons. To increase the signal intensities at codon 41, hybridization was repeated for a number of samples using hybridization temperatures of 38°C and 37°C instead of 39°C as recommended for the LIPA. This, however, caused a significant decrease of the test specificity, as seen by the appearance of nonspecific bands at the other codons.

Overall, only 48 (60%) of the serum samples showed hybridization reactions at all six codons covered by LIPA. Two of these samples are shown in Figure 1 (A and B). In 26 cases, one of the codons was undetermined by LIPA (Fig. 1C, D, F). In one serum sample two codons could not be evaluated (Fig. 1E); in five sera negative results were obtained at three or four codons covered by the LIPA (Fig. 1G). In all of these in part nonreactive samples the PCR had yielded clearly visible and specific amplified products, undistinguishable from those cases that were fully reactive at all codon positions.

Sequence Analysis of Genome Regions Surrounding the Resistance-Conferring Codons

The question was then addressed as to whether the hybridization failures of LIPA were all or in part caused by sequence variations. The sequence data within LIPA oligonucleotide probe binding regions were assessed in all cases. The results obtained from the samples that were not reactive in the LIPA are shown in Tables II, III, IV, V, and VI. Those mutations that were detected exclusively in the cases indeterminate in the LIPA are indicated in bold letters. The analysis of the sequence surrounding codon 41 (Table II) showed that eight samples had mutations in this part of the RT gene, which were not present in any of the LIPA-positive cases and may account for the hybridization failure of the HIV strains with the panel of oligonucleotide probes covering the codon 41 region. In two other codon 41 hybridization-negative specimens, there was a mixture of wild-type and mutant sequences detected by sequencing. The sequence data from the undetermined samples surrounding the other codon positions are shown in Tables III, IV, V, and VI. A

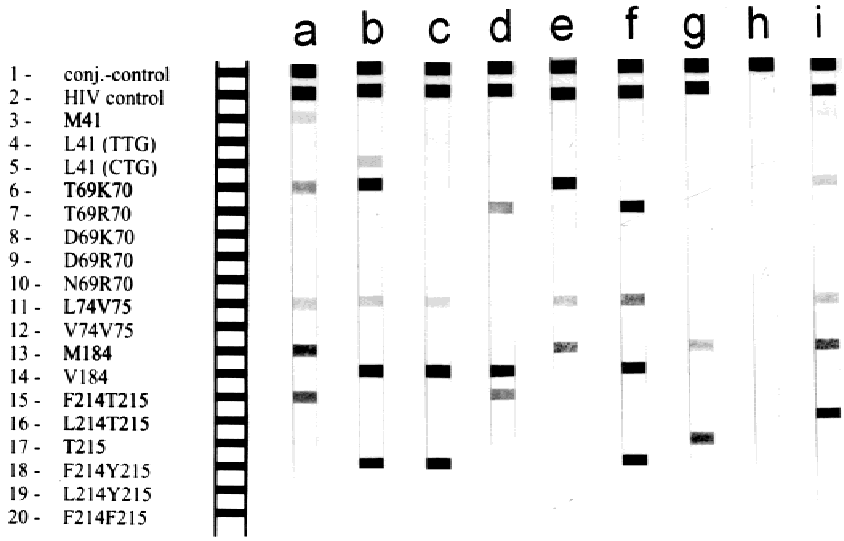

Fig. 1. Results obtained by LIPA testing from seven serum samples of HIV-1–infected patients. **A** and **B:** Samples yielding positive hybridization results at all codons included in the LIPA. A: Virus strain exhibiting hybridization bands on line 3, 6, 11, 13, and 15, which represent the wild-type sequences for codons 41, 69/70, 74, 184, and 215, respectively. B: virus strain showing resistance mutations at codons 41, 184, and 215, as visible from hybridization signals at the lines 5, 14, and 18. **C–G:** Samples showing various hybridization results and indeterminate results at individual codons of the LIPA. **H:** Negative control. **I:** Positive LIPA control.

TABLE II. Sequence Mutations Detected in Specimens Not Reactive in LIPA at Codon 41[a]

Codon	38 TGT	39 ACA	40 GAA	41 ATG	42 GAA	43 AAG
WT				T		
MU				C		
Serum samples (n = 20)						
n = 9[b]	–––	–––	–––	+––	–––	–––
n = 3[b]	–––	G––	–––	+––	–––	–––
n = 1	–––	**GA–**	––**T**	–––	–––	–––
n = 1	–––	**GA–**	–––	–––	––**G**	–––
n = 1	–––	–––	–––	T––	––**G**	–––
n = 2	–––	–––	–––	+––	–––	––**A**
n = 1	–––	–––	––G	C––	–––	**G**––
n = 1	–––	–––	–––	T––	–––	**C**––
n = 1	–––	–––	–––	T––	–––	**G**––

[a]Mutations printed in bold type were not detected in LIPA positive samples. + denotes WT or MU detected.
[b]One sample contained a mixture of WT/MU sequences.

TABLE III. Sequence Mutations Detected in Specimens Not Reactive in 4IPA at Codons 69/70[a]

Codon	68	69	70	71	72
WT	AGT	ACT	AAA	TGG	AGA
MU			G		
Serum samples (n = 4)					
n = 1	–––	–––	––**G**	–––	–––
n = 1	–––	–––	–G**G**	–––	–––
n = 1	G––	–––	–––	–––	–––
n = 1	––C	–––	–––	–––	––G

[a]Mutations printed in bold type were not detected in LIPA positive samples.

number of specific mutations that could have been responsible for the lack of hybridization were detected. However, there were a few cases in each of the RT gene regions where the undetermined LIPA results cannot be explained by the sequence data.

TABLE IV. Sequence Mutations Detected in Specimens Not Reactive in LIPA at Codon 74[a]

Codon	72	73	74	75	76
WT	AGA	AAA	TTA	GTA	GAT
MU			G		
Serum samples (n = 8)					
n = 2	––G	–––	–––	–––	–––
n = 3	–––	–––	––**G**	–––	–––
n = 1	–––	–––	**C**––	–––	–––
n = 1[b]	–––	–––	**A**––	–––	–––
n = 1	–––	–––	–––	–––	–––

[a]Mutations printed in bold type were not detected in LIPA positive samples.
[b]Mixture of MU and WT sequence.

TABLE V. Sequence Mutations Detected in Specimens Not Reactive in LIPA at Codon 184

Codon	182	183	184	185	186
WT	CAA/G	TAC	ATG	GAT	GAT
MU			G		
Serum samples (n = 6)					
n = 2	–––	––T	–––	–––	–––
n = 1	–––	––T	–––	–––	––C
n = 2	–––	–––	–––	–––	–––
n = 1	–––	––T	G––	–––	––C

Analysis of RT Resistance Mutations Not Covered by LIPA

Finally, the incidence of other RT gene mutations was assessed in our patients' samples, which are not covered by the LIPA but were also described to confer resistance against antiretroviral drugs and thus to be of possible importance for the clinical management of the patients. Mutations were sought at codons 67, 210, and 219, all shown to confer resistance to AZT [Larder et Kemp, 1989; Hooker et al., 1996], for mutations at

TABLE VI. Sequence Mutations Detected in Specimens Not Reactive in LIPA at Codon 215[a]

Codon	213	214	215	216	217
WT	GGA	TTT	ACC	ACA	CCA
MU			TT		
Serum samples (n = 7)					
n = 1	---	--**A**	---	---	---
n = 1	---	--**A**	TT–	---	--C
n = 1	--G	--C	TT–	---	---
n = 1	---	---	TA–	--C	---
n = 1	---	---	--**T**	--C	---
n = 1	---	---	---	--C	---
n = 1	---	---	**TTT**	---	---

[a]Mutations printed in bold type were not detected in LIPA positive samples.

codon 151, described to confer multidrug resistance to NIs [Shafer et al., 1994], and for mutations at codon 181, the most frequently observed mutation in the course of therapy with nonnucleoside inhibitors (NNIs) [Larder et al., 1994]. In 15 of the samples of this study, mutations were present at codon 67; in 6 cases there were mutations at codon 210; and in 5 cases mutations at codon 219 were found. In all these cases an AZT resistance was already predicted by at least one of the AZT resistance-specific mutations included in the LIPA. In none of the samples of this study was there a mutation at codon 151 detectable, while in four cases mutations at codon 181 were present, presumably due to previous therapy of the patients with NNIs.

It has been described that an M-to-V184 mutation may lead to a reversal of AZT resistance caused by mutations at codons 41 and 215 [Tisdale et al., 1993]. However, it was reported that the emergence of multiple AZT resistance mutations may lead to an AZT-resistant phenotype, even in the presence of the V184 mutation [Nijhuis et al., 1997]. Therefore, whenever the V184 mutation is present it may be important to screen for as many AZT resistance mutations as possible.

To assess the importance of these findings for the resistance diagnosis in our patients, 15 of the samples were examined, where the AZT resistance mutations at codons 215 and 41 were observed together with the V184 mutation. In six samples no other AZT resistance mutations were detected. In the other nine cases additional mutations were found at codon 67 and/or 210 and 219 and at codon 43, which has also been reported to contribute to the level of AZT resistance [Nijhuis et al., 1997].

DISCUSSION

The identification of drug-resistant HIV mutants is of increasing importance. The frequently observed development of viral resistance during antiretroviral therapy [Larder et al.,1989; St. Clair et al., 1991; Fitzgibbon et al., 1992; Tisdale et al., 1993], the observation that patients may already carry drug-resistant virus prior to initiation of the first treatment [Erice et al., 1993; Rubio et al., 1997], and the increasing number of drugs that can serve as therapeutic alternatives make a specific detection of HIV resistance against the individual therapeutic agents desirable. In the present study we have compared sequence analysis, which is usually applied in our laboratory for HIV drug resistance screening, to a recently developed RT-LIPA [Stuyver et al; 1997], which was described to facilitate genotypic HIV-1 resistance testing.

Testing by the LIPA resulted in a relatively large number of undetermined results, thus allowing a complete analysis of the six resistance codons in only 48 (60%) of the samples. It was apparent that this high rate of failure of hybridization reflected primarily a problem with the hybridization at the codon 41 wild-type or mutation probes, which was successful only in 75% of the samples. The detection rate determined in this study was significantly lower than that described for codon 41 by Stuyver et al. [1997], which was reported to be as high as 95.1% with European HIV-1–positive samples.

When sequence variations surrounding codon 41 were analyzed, it turned out that such variations in fact account for the nonreactivity with the hybridization probes in a number of the cases. Another explanation for the LIPA failure at codon 41 may in certain cases be the presence of a mixture of wild-type and mutant virus, which was detected in two nonreactive cases. Possibly, the simultaneous hybridization with two different probe cocktails led to signals that were too weak to be detected.

Unexpectedly, hybridization failure could not be explained by the sequence data in half of the codon 41-negative samples. The quality and quantity of the amplification products obtained from the nonreactive samples did not differ from those of the LIPA-positive specimens and usually yielded clearly positive results with the probes specific for the other codons. Thus, the hybridization at codon 41 seems to be less reliable under routine conditions than expected. In contrast, the detection rate at the other resistance codons covered by the LIPA was between 90% and 95% and is thus similar to the data published previously for European serum samples [Stuyver et al., 1997]. However, a somewhat lower rate of positivity at codons 184 and 215 has also been described by others [Schuurmann, 1997].

LIPA results, where they could be obtained, agreed well with sequence data, with the exception of one case at codon 70. It is likely that this discordant result may simply reflect the amplification of different subsets of the HIV populations present in the sample. In those cases where a mixed population of wild-type and mutant sequences was detected, LIPA and sequencing results were less consistent. It has been reported that the detection of minor populations present down to concentrations of 10% of the main population is possible by sequence analysis [Leitner et al., 1993]. However, it has not been described so far which concentration of minor variants may be detected by LIPA in the presence of a major wild-type population. In this study, LIPA indicated the presence of mutant strains in a wild-type population in a number of samples. This was

observed especially frequently at codon 69/70, where faint R70 mutant bands were detectable alongside a stronger wild-type signal. As the R70 mutant has been described to be usually the first sign of resistance development against AZT [Boucher et al., 1992; De Jong et al., 1996], a mutation arising in this region is of special interest. It was surprising, however, that the sequence data did not indicate a codon 70 mutant in any of these nine samples. It is not clear whether these findings simply reflect a higher sensitivity of the LIPA for detection of minor populations than the sequence analysis, or whether nonspecific reactions are observed in these cases. The fact that two of these patients had never received any antiretroviral therapy would more likely indicate that these faint bands indicating an additional R70 mutation are at least in part nonspecific. This is supported by the fact that four of the six cases where a mixture of wild-type and mutant virus at other codons was detected by LIPA were clearly confirmed by sequence data. Thus, further testings will be necessary to elucidate this aspect.

A major limitation of LIPA is the fact that it is designed for the detection of a relatively small number of the RT gene codons that are known to confer drug resistance, and that other regions, where mutations could also impair the viral drug sensitivity, are not included in the assay. It was shown previously that the detection of additional mutations conferring AZT resistance may be important to judge the extent of resistance against this drug, especially in the presence of a V184 mutation, which leads to a partial reversal of AZT resistance [Nijhuis et al., 1997]. According to those data, a reliable judgement of the resistance against AZT would not be possible in the presence of V184 from the identification of the AZT mutants covered by the LIPA alone. This was also the case in 15 of our patients, where a completely different AZT resistance background was observed by sequence analysis in the presence of V184 mutations.

During this study, one case was detected where a patient had obviously been infected by an already resistant HIV-1 strain. This was demonstrated by the identification of a codon 215 mutation in a therapy-naive patient. Similar findings have been published previously [Erice et al., 1993; Rubio et al., 1997], and these demonstrate the importance of analyzing the resistance profile in patients prior to their first antiretroviral therapy.

In summary, in this study a number of limitations of LIPA were observed when this assay was applied for routine testing. Thus, at the moment the RT-LIPA, although easy to handle and to interpret, is not a fully adequate substitute for sequence analysis for HIV resistance testing.

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